Meiji Co., Ltd.
1-2-10, Shinsuna, Koto-ku, Tokyo 136-8908, Japan
http://www.meiji.co.jp

January 9, 2012

Dr. Mary Ditto
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Ditto:

Re: GRAS Notification for *Propionibacterium freudenreichii* ET-3 Culture (Powder)

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the notifier [Meiji Co., Ltd., 1-2-10 Shinsuna, Koto-ku, Tokyo, 136-8908, Japan], a Notice of the determination, on the basis of scientific procedures, that *Propionibacterium freudenreichii* ET-3 Culture (Powder), produced by Meiji Co., Ltd., as defined in the enclosed documents, is GRAS under specific conditions of use as a food ingredient, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of *P. freudenreichii* ET-3 Culture (Powder) under the intended conditions of use, also are enclosed for review by the agency.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely, (b) (6)

Keisuke Seki
Business Development Dept. Team 1
International Business Unit
Meiji Co., Ltd.

000003

Meiji Co., Ltd.
1-2-10, Shinsuna, Koto-ku, Tokyo 136-8908, Japan
http://www.meiji.co.jp

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Dr. Mary Ditto
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Ditto:

Re: GRAS Notification for *Propionibacterium freudenreichii* ET-3 Culture (Powder)

Please be advised that Intertek Cantox is representing Meiji Co., Ltd. and is fully authorized to act on our behalf with respect to the Generally Recognized as Safe (GRAS) notification for *Propionibacterium freudenreichii* ET-3 Culture (Powder) submitted on January 9, 2012.

Sincerely,

Keisuke Seki
Business Development Dept. Team 1
International Business Unit
Meiji Co., Ltd.
(b) (6)

cc: Dr. Andrea W. Wong, Intertek Cantox

GRAS Exemption Claim for *Propionibacterium freudenreichii* ET-3 Culture (Powder)

Submitted for:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835
USA

Submitted by:

Meiji Co., Ltd.
1-2-10, Shinsuna
Koto-ku, Tokyo 136-8908
Japan

January 9, 2012

GRAS Exemption Claim for *Propionibacterium freudenreichii* ET-3 Culture (Powder)

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I GRAS EXEMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997) (U.S. FDA, 1997)]

Propionibacterium freudenreichii ET-3 culture (powder) has been determined to be Generally Recognized as Safe (GRAS) by Meiji Co., Ltd. (Meiji) for use in a variety of traditional food products in the United States (U.S.), consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections. Therefore, the use of *P. freudenreichii* ET-3 culture (powder) in foods as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Hirotada Nakamura,
General Manager of Business Development Dept.,
International Business Unit

January 9th, 2012
Date

I.B Name and Address of Notifier

Keisuke Seki
Meiji Co., Ltd.
1-2-10, Shinsuna
Koto-ku, Tokyo 136-8908
Japan

I.C Common Name of the Notified Substance

The common name of the notified substance is *P. freudenreichii* ET-3 culture (powder).

I.D Conditions of Intended Use in Food

I.D.1 Intended Uses of *P. freudenreichii* ET-3 Culture (Powder) and Levels of Use

P. freudenreichii ET-3 culture (powder) comprises two thirds *P. freudenreichii* ET-3 solid culture and one third potato and modified starches used for texture purposes. Meiji intends to market *P. freudenreichii* ET-3 culture (powder), produced from whey powder fermented by *P. freudenreichii* ET-3, as a food ingredient for use at levels providing up to 15% *P. freudenreichii*

ET-3 solid culture in selected food categories. The ingredient is not intended for use in infant foods or formula. The individual proposed food-uses and maximum use-levels for *P. freudenreichii* ET-3 solid culture are summarized in Table I.D.1-1.

Table I.D.1-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *P. freudenreichii* ET-3 Solid Culture in the U.S.

Food Category	Proposed Food-Uses	RACC* (g or mL)	*P. freudenreichii* ET-3 Solid Culture Level (g/serving*)	Use-Levels (%)
Beverages and Beverage Bases	Carbonated beverages	240 mL	0.2	0.083
	Enhanced waters	240 mL	0.4	0.17
	Non-Milk based Meal replacements	240 mL	0.5	0.21
Breakfast Cereals	RTE cereals	55 g (granola) 30 g (regular) 15 g (puffed)	0.3	0.54 (granola) 0.86 (regular) 1.2 (puffed)
Cheeses	Natural and hard cheeses	55 g 5 g (grated)	0.4	0.73 1.34 (grated)
Coffee and Tea**	Coffee	240 mL	0.3	0.12
	Tea	240 mL	0.3	0.12
Fats and Oils	Butter	15 mL	0.3	2
Frozen Dairy Desserts and Mixes	Frozen Yogurt	120 g	0.3	0.24
	Ice cream	120 g	0.3	0.24
Gelatins, Puddings, and Fillings	Custard pudding	125 g	0.3	0.21
	RTE Gelatin desserts	125 g	0.3	0.3
Grain Products and Pastas	Meal replacement bars	40 g	0.4	1
Milk Products	Flavored milk and milk drinks	240 mL	0.4	0.17
	Milk-based meal replacements	240 mL	0.4	0.17
	Powdered flavored milk drinks	2 g	0.3	15
	Yogurt	225 g	0.4	0.18
Processed Fruits and Fruit Juices	Fruit-Flavored Drinks and Ades	240 mL	0.3	0.12
	Fruit juice	240 mL	0.3	0.12
	Nectar	240 mL	0.4	0.17
Processed Vegetables and Vegetable Juices	Vegetable juice	240 mL	0.6	0.25
Soft Candy	Chocolate	40 g	0.4	1

RTE = Ready-to-eat.
*RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12 – U.S. FDA, 2011). When a range of values is reported for a proposed food-use, particular foods within that food-use may differ with respect to their RACC.
**All representative food codes in this category were selected to provide a conservative estimate of intake; however, the ingredient will not be used in some applications within this category (*e.g.*, leaf tea).

I.D.2 Estimated Consumption of *P. freudenreichii* ET-3 Culture (Powder) Based upon Intended Uses

Estimates for the intake of *P. freudenreichii* ET-3 solid culture were based on the intended food uses and use levels in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2003-2004 and 2005-2006 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2006, 2009; USDA, 2009).

The percentage of the total U.S. population identified as potential consumers was 96.8% (16,155 actual users identified). Consumption of all proposed food uses, calculated according to the assumptions described above, by the total U.S. population resulted in estimated mean all-person and all-user intakes of *P. freudenreichii* ET-3 solid culture of 1.6 g/person/day (27 mg/kg body weight/day) for both values. The 90th percentile all-person and all-user intakes of *P. freudenreichii* ET-3 solid culture from all proposed food uses by the total population were 2.8 g/person/day for both values (50 mg/kg body weight/day).

On an individual population basis, the greatest mean all-user intakes of *P. freudenreichii* ET-3 solid culture were determined to occur in male adults, at 2.0 g/ person/day, or 23 mg/kg body weight/day, respectively. Children displayed the lowest mean all-person and all-user intakes of *P. freudenreichii* ET-3 solid culture, with values of 1.2 g/person/day for both intake values. On a body weight basis, mean all-person intakes of *P. freudenreichii* ET-3 solid culture were highest in children, with values of 47 mg/kg body weight/day. The highest mean all-user per kilogram body weight intake of *P. freudenreichii* ET-3 solid culture was observed in children, at 47 mg/kg body weight/day, while the lowest mean all-person and all-user intakes on a per kilogram body weight basis were observed in female teenagers and adults, with a value of 22 mg/kg body weight/day for both intake values and population groups.

When heavy consumers (90th percentile) were assessed, all-person and all-user intakes of *P. freudenreichii* ET-3 solid culture from all proposed food-uses also were determined to be greatest in male adults (3.3 g/person/day, for both values). The lowest 90th percentile all-person and all-user intakes were observed in female teenagers, with intakes of 2.0 g/person/day for both values. On a body weight basis, children were determined to have the greatest all-person and all-user 90th percentile intakes of *P. freudenreichii* ET-3 solid culture, with values of 82 mg/kg body weight/day for both values. The lowest all-person and all-user 90th percentile intakes of *P. freudenreichii* ET-3 solid culture on a body weight basis were observed in female teenagers (36 mg/kg body weight/day for both values).

This type of intake methodology is generally considered to be "worst case" (*i.e.*, more likely to err on the side of overestimation, rather than underestimation, of actual intakes) as a result of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects

the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are consumed relatively infrequently.

I.E Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2011), *P. freudenreichii* ET-3 culture (powder) has been determined to be GRAS on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of *P. freudenreichii* ET-3 culture (powder), as discussed herein, and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of the *P. freudenreichii* ET-3 culture (powder) ingredient as a component of food [see Appendix A, entitled "Expert Panel Consensus Statement Concerning the GRAS Status of *Propionibacterium freudenreichii* ET-3 Culture (Powder) for Use in Foods"].

At the request of Meiji, an Expert Panel ("the Expert Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether the intended uses of *P. freudenreichii* ET-3 culture (powder) in foods are safe and suitable and would be GRAS based on scientific procedures.

The Panel consisted of the following qualified scientific experts: Professor Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Professor John A. Thomas, Ph.D. (Indiana University School of Medicine), and Professor Eric A. Johnson, Sc.D. (University of Wisconsin-Madison).

The Expert Panel convened on behalf of Meiji independently and collectively, and critically evaluated the data and information summarized herein and concluded that the intended uses in traditional foods described herein for *P. freudenreichii* ET-3 culture (powder), meeting appropriate food-grade specifications and manufactured according to current Good Manufacturing Practice (cGMP), are safe, suitable, and GRAS based on scientific procedures. It also is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion.

P. freudenreichii ET-3 culture (powder) is GRAS based on scientific procedures for its intended use as a food ingredient; therefore, it is excluded from the definition of a food additive, and thus, may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

Meiji Co. Ltd.
1-2-10, Shinsuna
Koto-ku, Tokyo 136-8908
Japan

Should FDA have any questions or additional information requests regarding this notification, Meiji will supply these data and information.

II. DETAILED INFORMATION ABOUT THE SOURCE AND IDENTITY OF THE SUBSTANCE

II.A Source and Identity

P. freudenreichii ET-3 is isolated from Emmental cheese produced in Switzerland.

P. freudenreichii ET-3 culture (powder) (trade name: Bifivital or BGS) is yellow to reddish-brown in color with no remarkable taste or odor. The composition of the product is outlined below in Table II.A-1.

Table II.A-1 Composition of *P. freudenreichii* ET-3 Culture (Powder)

Protein (g/100 g)	10
Fat (g/100 g)	0.5
Carbohydrates (g/100 g)	70
Ash (g/100 g)	16
Calcium (mg/100 g)	350
Magnesium (mg/100 g)	80
Potassium (mg/100 g)	4,600
Sodium (mg/100 g)	1,000
Phosphorus (mg/100 g)	440

II.B Method of Manufacture

A schematic diagram of the general manufacturing process employed to produce *P. freudenreichii* ET-3 culture (powder) is presented in Figure II.B-1. In the first step, whey powder undergoes proteolysis, which is catalyzed by a protease. Following proteolysis, brewing

yeast extract and ammonium sulfate are added to the solution. The solution is then filtered, sterilized at 140°C for ≥4 seconds, and re-filtered. The solution is then sent to the fermenter.

The fermentation medium described above undergoes 4 serial fermentations with *P. freudenreichii* ET-3. Each fermentation step is allowed to proceed for 48 to 96 hours at 35 to 37°C, during which pH is maintained at approximately 6 with potassium carbonate. During the fermentation steps, 1,4-dihydroxy-2-naphthoic acid (DHNA) is produced by *P. freudenreichii* ET-3. The fermentation is terminated upon cessation of the requirement for pH control, which is assessed by monitoring the alkali consumption of the culture solution. This solution is then refrigerated for 5 to 10 hours, during which pH remains at or below 5.6. Sodium ascorbate is added to the solution to stabilize the activity of DHNA. The solution is then filtered, heated to 70°C for ≥5 minutes, and then cooled to 50 to 55°C. Potato starch and modified starch are added to the solution, which is then heated to 80°C for ≥5 minutes to allow gelatinization to occur, during which the *P. freudenreichii* ET-3 cells are killed[1]. The solution is then freeze-dried, milled, sieved, subjected to a magnet to remove metals, weighed, and packaged. As mentioned above, the final *P. freudenreichii* ET-3 culture (powder) product contains two thirds *P. freudenreichii* ET-3 solid culture and one third potato and modified starches. The ingredients used in the production of *P. freudenreichii* ET-3 culture (powder) remain in the final product.

All raw materials and processing aids used in the manufacture of *P. freudenreichii* ET-3 culture (powder) are used in compliance with appropriate federal regulations as indicated in Table II.B-1.

[1] Meiji has indicated that the final product contains <300 CFU live *P. freudenreichii* ET-3/g.

Figure II.B-1 **Schematic Overview of the Manufacturing Process for *P. freudenreichii* ET-3 Culture (Powder)**

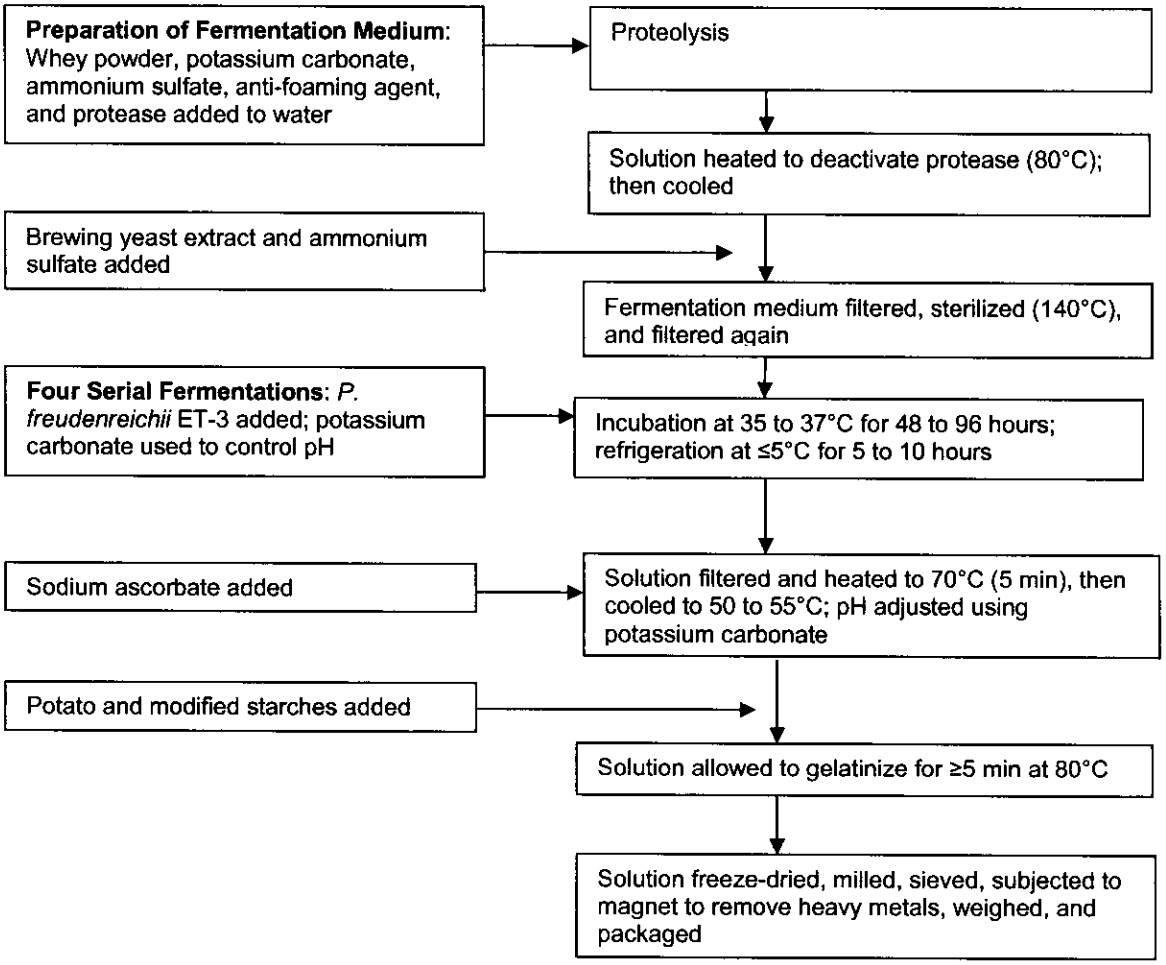

Table II.B-1 List of Ingredients, Processing Aids, and Equipment Used in the Manufacture of *P. freudenreichii* ET-3 Culture (Powder)

Component	Function	Reference to U.S. Regulatory Status
Whey powder	Component of fermentation medium	21 CFR §184.1979 (U.S. FDA, 2011)
Anti-foaming agent	Used in preparation of fermentation medium	21 CFR §172.854 and 172.859 (U.S. FDA, 2011)
Potassium carbonate	pH control agent	21 CFR §184.1619[a] (U.S. FDA, 2011)
Ammonium sulfate	Source of nitrogen for *P. freudenreichii* ET-3 bacteria	21 CFR §184.1143 (U.S. FDA, 2011)
Protease	Used to break down whey protein	GRN 000090 (U.S. FDA, 2002)
Brewing yeast extract	Component of fermentation medium	21 CFR §184.1983 (U.S. FDA, 2011)
Precision etching technology (PET) filter	Purification	[b]
Sodium ascorbate	Used to stabilize activity of DHNA	21 CFR §182.3731 (U.S. FDA, 2011)
Potato starch	Filler/texturizer	21 CFR §101.12 (U.S. FDA, 2011)
Modified starch	Filler/texturizer	21 CFR §172.892 (U.S. FDA, 2011)

CFR = Code of Federal Regulations; DHNA = 1,4-dihydroxy-2-naphthoic acid ; FDA = Food and Drug Administration; GRN = GRAS notice.

[a] Although this processing aid may not meet the lead and "loss on drying" specifications of the Food Chemicals Codex (FCC) [noted specifications are ≤20 ppm for heavy metals (as lead) (FCC requires ≤2 ppm), and ≤2% for loss on drying (FCC requires ≤1%)], the final product [*P. freudenreichii* ET-3 culture (powder)] meets all appropriate food-grade specifications. Thus, the lead and moisture content for potassium carbonate are not of concern.

[b] This processing aid is not listed in 21 CFR; however, it is expected that the use of this stainless steel filter will be acceptable.

II.C Specifications and Analytical Data

P. freudenreichii ET-3 culture (powder) is produced in accordance with cGMP, and in order to ensure a consistent and safe product, Meiji has established food-grade specification parameters for the final ingredient. The product specifications for *P. freudenreichii* ET-3 culture (powder) are presented in Table II.C-1.

Analyses of 4 non-consecutive lots of *P. freudenreichii* ET-3 culture (powder) confirm that the manufacturing process results in a product that is consistent and complies with product specifications. The analytical data also demonstrate the absence of chemical impurities or microbiological contamination. Complete certificates of analysis for these lots are provided in Appendix B.

Meiji has tested several lots of *P. freudenreichii* ET-3 culture (powder) for the presence of heat-resistant organisms (*i.e.*, bacteria able to grow at 55°C, including *Bacillus* sp., *Lactobacillus* sp., *Thermus* sp., and *Thermomicrobium* sp.). Results confirm that heat-resistant bacteria are not produced during the fermentation process (data not shown).

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Table II.C-1 Product Specifications for *P. freudenreichii* ET-3 Culture (Powder)

Specification Parameter	Specification	Method
Physical and Chemical Parameters		
Taste	No abnormal taste or odor	Organoleptic
Color	Yellow to reddish-brown	Visual
Moisture	≤8.0%	Food Sanitation Inspection Guidelines in Japan (dry at 98°C for 5 hours)
Protein	10.0 ± 2.0%	Food Sanitation Inspection Guidelines in Japan (Kjeldahl method; conversion factor: 6.25)
Ash	16.0 ± 3.0%[a]	Food Sanitation Inspection Guidelines in Japan (dry at 550°C for 5 hours)
Lead	≤1 ppm	Food Sanitation Inspection Guidelines in Japan (AAS)
DHNA concentration	20 to 90 µg/g	HPLC[b]
Microbiological Parameters		
Total viable bacteria count	≤10,000 CFU/g	Food Sanitation Inspection Guidelines Standard methods in Japan (SMA)
Coliforms	Negative	Food Sanitation Inspection Guidelines in Japan (BGLB method)
Staphylococcus aureus	Negative	Food Sanitation Inspection Guidelines in Japan (Mannitol salt agar)
Fungi and yeast	≤1,000 CFU/g	Food Sanitation Inspection Guidelines in Japan (Potato dextrose agar)
Salmonella sp.	Negative	AOAC method (17.09.02 *Salmonella* in processed foods 967.26)

AAS = atomic absorption spectrophotometry; AOAC = Association of Official Analytical Chemists (AOAC, 2000[2]); BGLB = brilliant green lactose bile; CFU = colony-forming units; DHNA = 1,4-dihydroxy-2-naphthoic acid; HPLC = high performance liquid chromatography; SMA = standard method agar.
[a] Meiji has indicated that the remainder of the weight of *P. freudenreichii* ET-3 culture (powder) (*i.e.*, the remaining 66 to 71% after accounting for moisture, protein, and ash content) is composed of primarily of carbohydrates, with lipids contributing approximately 0.5%.
[b] HPLC method is described in Appendix C.

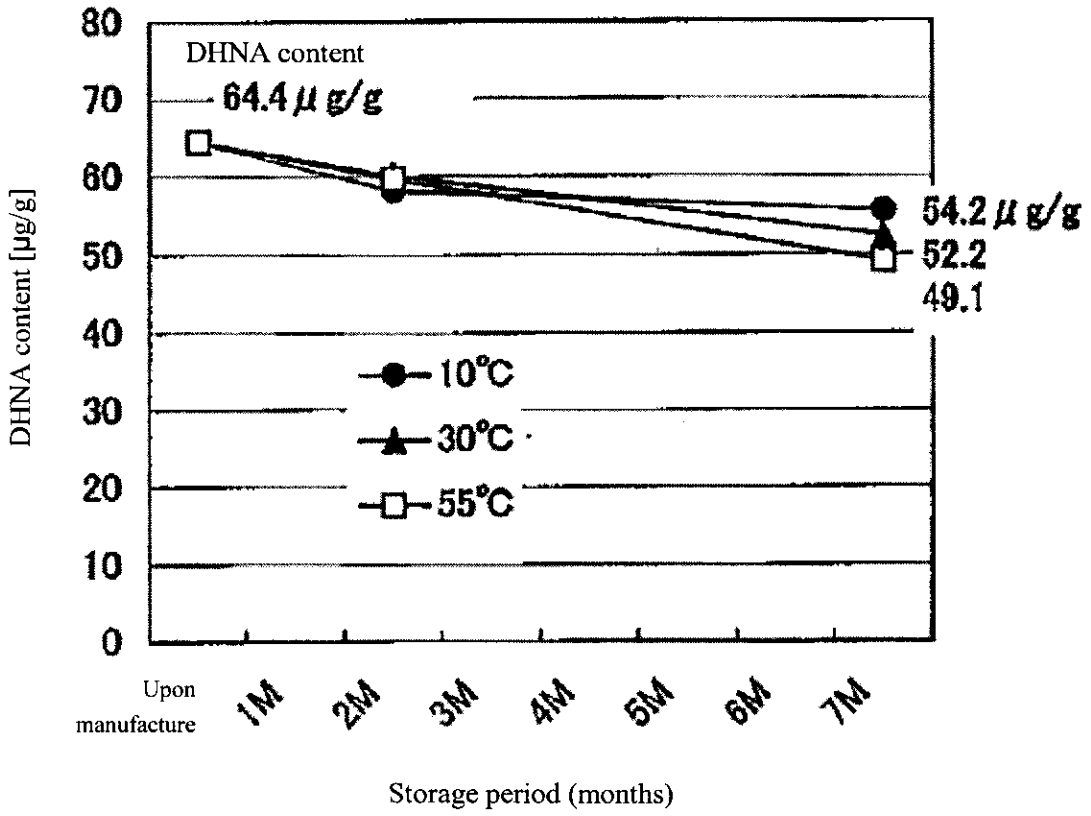
II.D Stability of *P. freudenreichii* ET-3 Culture (Powder)

Stability testing conducted on *P. freudenreichii* ET-3 culture (powder) has demonstrated that the product is stable for up to 14 months at room temperature. DHNA content was reported to be 97.5 and 86.3% of initial values after 7 and 14 months of storage, respectively. Stability testing also was conducted for 7 months at varying temperatures (10, 30, and 55°C). As demonstrated in Figure II.D-1, the DHNA content remained within specifications (20 to 90 µg/g) under all storage conditions. Additional stability tests provided by Meiji have indicated that the DHNA content of *P. freudenreichii* ET-3 culture tablets remained stable following storage in an

[2] AOAC (2000). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 17th edition (2002, Revision 1)*. Arlington (VA): Association of Official Analytical Chemists (AOAC).

unsealed container with a desiccant at 30 or 40°C for 12 months, or 25°C for 15 months (DHNA concentrations measured at 12 and 15 months were equal to those measured at baseline).

Figure II.D-1 **DHNA Content of *P. freudenreichii* ET-3 Culture (Powder) Under Various Storage Conditions**



III. SELF-LIMITING LEVELS OF USE

No characteristics of *P. freudenreichii* ET-3 culture (powder) were identified that would be expected to limit its use.

IV. BASIS FOR GRAS DETERMINATION

IV.A Documentation to Support the Safety of *P. freudenreichii* ET-3 Culture (Powder)

The determination that *P. freudenreichii* ET-3 culture (powder) is GRAS is based on scientific procedures, and the information supporting the general recognition of the safety of the ingredient includes:

- Data pertaining to the identity, intended use, and estimated intake of *P. freudenreichii* ET-3 solid culture;
- The natural occurrence of *Propionibacteria*, including *P. freudenreichii*, in food and documented history of safe use;
- The regulatory status of *Propionibacteria* in the U.S. and commercial availability of *P. freudenreichii* ET-3 culture food products in Japan;
- The lack of systemic exposure to *P. freudenreichii* ET-3 and metabolic fate of DHNA[3], which is secreted from *P. freudenreichii* ET-3 during fermentation; and
- Toxicological and human studies conducted with *P. freudenreichii* ET-3 culture (in powder, tablet, or solution form) or DHNA.

Moreover, these data were reviewed by a panel of experts, qualified by scientific training and experience to evaluate the safety of ingredients as components of food, who concluded that the intended uses of *P. freudenreichii* ET-3 culture (powder) are safe and suitable and would be GRAS based on scientific procedures [see Appendix A, entitled "Expert Panel Consensus Statement Concerning the GRAS Status of *Propionibacterium freudenreichii* ET-3 Culture (Powder) for Use in Foods"]. A summary of these data is presented herein.

IV.B Regulatory Status, Natural Occurrence, and Background Dietary Consumption of *P. freudenreichii* ET-3 and *P. freudenreichii* ET-3 Culture

In Japan, *P. freudenreichii* ET-3 culture milk drinks and tablets have been commercially available since 2001, and were granted Foods for Specified Health Use (FOSHU) status in 2001 and 2002 (respectively). Additionally, *P. freudenreichii* ET-3 culture powder became commercially available in Japan in 2003 (MHLW, 2007[4]). *P. freudenreichii* is permitted for use in the U.S. as a starter culture in the production of Swiss, Emmentaler, and Gruyere cheeses (Meile *et al.*, 2008). In addition, the FDA has issued letters of no objection in response to 2 GRAS Notices pertaining to anti-microbial agents produced *via* the fermentation of corn, cane, or beet sugar, skim milk, or dextrose by *P. freudenreichii shermanii* (GRN 000128 and GRN 000240) (U.S. FDA, 2003, 2008). Meile *et al.* (2008) concluded in their safety assessment of dairy microorganisms that *Propionibacteria*, including *P. freudenreichii,* and *Bifidobacterium* species in dairy foods generally do not present any significant health hazards.

As mentioned above, *P. freudenreichii* ET-3 is isolated from Emmental cheese. As such, this strain has a long history of use in commonly consumed foods.

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[3] DHNA is considered by Meiji to be the primary component of *P. freudenreichii* ET-3 culture (powder) responsible for the bifidogenic effects of the ingredient.

[4] MHLW (2007). *Food for Specified Health Uses (FOSHU).* Tokyo, Japan: Ministry of Health, Labor and Welfare, Japan (MHLW). Available at: http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html.

Due to its long history of safe use in foods, the European Food Safety Authority (EFSA) has granted Qualified Presumption of Safety (QPS) status to *P. freudenreichii* (EFSA, 2007). Granting of QPS status indicates that the evaluation of a defined taxonomic group for use in food and feed production by EFSA did not raise safety concerns or, if safety concerns existed, they could be defined and excluded. In addition, the International Dairy Federation, in collaboration with the European Food and Feed Cultures Association, has included *P. freudenreichii* on its inventory of microorganisms with a documented history of use in human food without adverse effects (Mogensen *et al.*, 2002).

IV.C Metabolic Fate of *P. freudenreichii* ET-3 Culture (Powder)

During the heating step in the manufacturing process for *P. freudenreichii* ET-3 culture (powder), *P. freudenreichii* ET-3 bacteria are killed. As the intestinal mucosal barrier is impermeable to bacteria in healthy individuals, any remaining intact bacteria (live or dead) are not expected to be absorbed from the gastrointestinal tract. Instead, *P. freudenreichii* ET-3 is expected to transit through the gastrointestinal tract and be excreted in the feces.

Bacterial translocation (*i.e.*, the escape of viable bacteria from the gastrointestinal tract) is an unusual effect that may occur in individuals whose gastrointestinal integrity is compromised, the most common results of which include bacterial transportation to the mesenteric lymph nodes, liver, spleen, and general circulation, potentially resulting in bacteremia, sepsis, and multiple organ failure (Ishibashi and Yamazaki, 2001; Lichtman, 2001; MacFie, 2004; Liong, 2008). Given that *P. freudenreichii* ET-3 culture (powder) contains very little live bacteria (*i.e.*, <300 CFU *P. freudenreichii* ET-3/g), bacterial translocation and its associated systemic effects would be highly unlikely to occur in healthy individuals. DHNA, which is secreted during the fermentation process, appears to be largely resistant to digestion under the acidic conditions of the stomach. In a study conducted by Meiji, *P. freudenreichii* ET-3 culture (solution) was incubated in artificial gastric juice for up to 4 hours [Meiji, 2002 (unpublished)]. The bifidobacteria growth stimulating activity of the ingredient was 66.5% of baseline levels after 2 hours of incubation and 54.8% after 4 hours of incubation.

The low molecular weight of DHNA (240.18 g/mol) favors its absorption *via* the gastrointestinal tract. Data regarding other naphthoic acids structurally similar to DHNA suggest that DHNA is absorbed following oral exposure. The low molecular weight (188.18 g/mol) and moderate lipophilicity ($\log_P$ = 3.29) of 1-hydroxy-2-naphthoic acid supports its absorption *via* the gastrointestinal tract (ECHA, 2008). Due to the presence of an additional hydroxyl group on DHNA, it is likely to be slightly less volatile and more water soluble than mono-hydroxylated naphthoic acids. Miller *et al.* (2010) noted that the high lipophilicity and relatively strong acidity of 1-hydroxy-2-naphthoic acid make it a useful compound to increase the intestinal absorption of anti-viral medications with inherently poor bioavailability.

In a study designed to develop a method for the determination of 1-hydroxy-2-naphthoic acid in human plasma, 6 healthy male volunteers consumed a single oral dose of 500 µg salmeterol xinafoate (the 1-hydroxy-2-naphthoic acid salt of salmeterol, a β2 adrenergic agonist bronchodilator) containing 225 µg 1-hydroxy-2-naphthoic acid (Chilton *et al.*, 1995). Blood samples were taken at baseline, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours post-dose, and then twice weekly for the following 5 weeks. Maximum plasma 1-hydroxy-2-naphthoic acid concentrations were observed 1 hour post-dose, and ranged from 35.3 to 66.8 ng/mL. Although plasma half-life was not reported, minimum plasma 1-hydroxy-2-naphthoic acid concentrations (*i.e.*, approximately 20 ng/mL plasma) were observed 24 hours post-dose (after which point no plasma 1-hydroxy-2-naphthoic acid concentration data were reported). Chilton *et al.* (1995) commented that upon absorption into the systemic circulation, 1-hydroxy-2-naphthoic acid had no apparent pharmacological activity, and was highly (*i.e.*, >99%) protein-bound.

The available physical and chemical data regarding DHNA, and human data regarding 1-hydroxy-2-naphthoic acid, suggest that a portion[5] of orally administered DHNA will be absorbed following oral exposure, and that the majority of the absorbed dose may be eliminated within 24 hours of absorption (based on the observation of minimum plasma concentrations of 1-hydroxy-2-naphthoic acid in human plasma 24 hours following oral exposure). No data pertaining to the metabolism of DHNA or similar compounds were identified.

IV.D Toxicological Studies

IV.D.1 Animal Studies

Two published animal studies investigating the effects of *P. freudenreichii* ET-3 culture (powder or solution) on rats, 9 and 28 days in duration, were identified in the literature (Uchida and Mogami, 2005; Uchida *et al.*, 2011a). In addition, several unpublished animal studies assessing the safety of *P. freudenreichii* ET-3 culture (powder or solution) or the effects of DHNA following oral exposure were evaluated. No acute, reproductive, or developmental toxicity studies or carcinogenicity studies pertaining to oral exposure to *P. freudenreichii* ET-3 culture or DHNA were identified.

IV.D.1.1 *P. freudenreichii ET-3 Culture Studies*

A 28-day toxicity study was conducted in which groups of 6-week-old male and female Sprague-Dawley rats (n=12/sex) were administered powdered *P. freudenreichii* ET-3 solid culture by gavage at doses of 0 (control), 0 (placebo), or 6,000 mg/kg body weight/day (active group; dose corresponds to 576 µg DHNA/kg body weight/day) (Uchida *et al.*, 2011a). Doses of

[5] Using an average human plasma volume of 0.04 L/kg body weight (Katzung, 2004), the administered dose of 225 µg 1-hydroxy-2-naphthoic acid, and peak plasma 1-hydroxy-2-naphthoic acid concentrations of 35.3 to 66.8 ng/mL, the average absorption of 1-hydroxy-2-naphthoic acid in this study would be approximately 37.6 to 71.1% of the administered dose (for a 60 kg individual).

P. freudenreichii ET-3 solid culture administered were calculated based on the individual body weight of each animal. The control group received the vehicle (water), while the placebo group received a powdered whey culture medium formulation in water.

There were no deaths or signs of toxicity observed over the duration of the study, nor were there any significant differences between groups with respect to body weight or food consumption. Rats (1 to 2) in all groups were reported to have persistent intravitreal blood vessels, specifically unilateral or bilateral hyaloid arteries, following ophthalmological examinations. This effect was reported to be a normal occurrence during ocular development, and was not considered to be compound-related.

Examination of hematological parameters revealed significantly increased mean corpuscular hemoglobin concentration (MCHC) (placebo females *vs.* control females) and reticulocyte ratio (active males *vs.* placebo males). Clinical chemistry analysis demonstrated significant decreases in glucose and sodium concentrations (placebo males *vs.* control males), increased BUN concentration (active males *vs.* placebo males), and decreased creatinine concentration (active females *vs.* placebo females). Since the observed changes occurred only in 1 sex and were very slight in magnitude[6], they were not considered to be compound-related. No other differences in hematology or clinical chemistry parameters were reported. Additionally, there were no significant differences in prothrombin time, activated partial thromboplastin time, or fibrinogen concentration.

Mean urinary pH was reported to be significantly increased in males and females in the active group relative to placebo, and was significantly decreased in placebo males compared to control males and unaffected in placebo females. Although the observed increase in the active group was statistically significant, it was slight in magnitude and remained within the range of pH values observed in control animals. Thus, it was not considered to be test article-related and was at least partially attributed to the observed decrease in urinary pH in the placebo group. Significantly fewer males in the active group tested positive for urobilinogen, and significantly fewer females in the active group tested positive for urinary ketone bodies, compared to the male and female placebo groups, respectively. A significantly higher number of female rats tested positive for urinary protein (placebo group compared to control and active group compared to placebo). However, no corresponding biochemical or physiological indications of kidney toxicity were observed; thus, increased urinary protein concentrations were attributed to a physiological response to increased dietary protein rather than adverse renal effects. Total daily excretion of potassium also was increased in male and female rats in the active group compared to placebo, although this increase was considered to be a physiological response to the observed slight urinary alkalinization. Additionally, plasma potassium levels did not significantly differ between the active and placebo groups. No differences in other electrolytes,

[6] Although the between-group differences in hematological and clinical chemistry were statistically significant, the differences remained within historical control ranges.

water intake, urinary output, or osmotic pressure were reported for the treatment group (compared to placebo). The observed changes in urinalysis parameters were reported to be marginal, and as they generally occurred in 1 sex and were not accompanied by any gross or histopathological changes that would be suggestive of a significant adverse effect, were not considered compound-related.

Significantly increased absolute (but not relative) seminal vesicle weight was observed in males in the placebo group (compared to controls). Absolute and relative thymus weights were non-significantly decreased in placebo females; however, no significant difference in absolute and relative thymus weights and no gross or histological abnormalities between active and control females were reported. Gross findings at necropsy occurred at similar frequencies in control and active animals and were considered to be incidental.

Histological observations included mild thickening of the limiting ridge of the stomach in most rats of both sexes in the active and placebo groups but not in the control group. The authors reported that similar thickening was not observed in the esophagus and forestomach, areas of the gastrointestinal tract that share a stratified squamous epithelium. Furthermore, since the limiting ridge is specific to rodents, this observation was considered not to be toxicologically relevant to humans. Mild cell infiltration in the cecal lamina propria of the appendix was observed at a greater incidence in males in both the active and placebo groups compared to the control group and at a similar incidence in females in all groups. Since this is a common physiological change and was observed in the control group, it was not considered compound-related. Other histological findings occurred at low rates and were considered incidental. The authors of this study concluded that "neither *P. freudenreichii* ET-3 culture nor the whey culture medium used in the production of the ingredient induced any compound-related adverse effects in Sprague-Dawley rats each at a dose of 6,000 mg/kg body weight/day."

An additional animal study was conducted to assess the effects of *P. freudenreichii* ET-3 culture, unfermented whey culture medium, propionic acid, or acetic acid on the healing of induced colitis in male Sprague-Dawley rats (Uchida and Mogami, 2005). Following induction of colitis *via* luminal injection with 2,4,6-trinitrobenzene sulfonic acid (TNBS), rats were administered *P. freudenreichii* ET-3 culture (powder) (at doses of 2,000 or 6,000 mg/kg body weight/day; equivalent to 1,000 or 3,000 mg *P. freudenreichii* ET-3 solid culture, or 82.3 to 372.6 µg DHNA, per kg body weight/day), whey culture medium (2,000 mg/kg body weight/day), sodium propionate (10 mL of a 0.1 M solution/kg body weight/day), sodium acetate (10 mL of a 0.1 M solution/kg body weight/day), or distilled water (10 mL/kg body weight/day) orally for 9 days. On the 10th day after the induction of colitis, rats were killed and their colons excised, and the size and morphology of TNBS-induced ulcers were examined. It was reported that *P. freudenreichii* ET-3 culture and sodium propionate had no effects indicative of toxicity on ulcer size and morphology. The results of this study suggest that *P. freudenreichii* ET-3 culture

(powder) would not cause adverse effects to the intestinal epithelium even under colonic disease conditions.

IV.D.1.2 *Unpublished P. freudenreichii ET-3 Culture Studies*

Six-week-old specific pathogen-free (SPF) male and female Sprague-Dawley rats were administered 2,500, 5,000,or 10,000 mg *P. freudenreichii* ET-3 culture solution/kg body weight/day (providing approximately 20 to 39, 40 to 60, or 80 to 120 µg DHNA/kg body weight/day[7] and doses equivalent to 250, 500, or 1,000 mg *P. freudenreichii* ET-3 solid culture/kg body weight/day, respectively) by gavage for 4 weeks (Nishimura *et al.*, 1999 [unpublished]). No adverse effects with respect to general condition, body weight, ophthalmology, urinalysis, hematology, or pathology were observed, and the no-observed-adverse-effect level (NOAEL) for *P. freudenreichii* ET-3 culture solution in rats was determined to be 10,000 mg/kg body weight/day [*i.e.,* approximately 80 to 120 µg DHNA/kg body weight/day, or 1,000 mg *P. freudenreichii* ET-3 solid culture/kg body weight/day], the highest dose tested.

IV.D.1.3 *DHNA Studies*

Matsubara *et al.* (2010) investigated the effects of DHNA on bone resorption in male ICR mice with immunosuppressant-induced osteoporosis. Thirty 6-week-old male ICR mice were intraperitoneally administered an immunosuppressant (FK506, also known as Tacrolimus) with or without oral administration of 1 mg DHNA/kg body weight/day, or vehicle (control) for 5 weeks. The authors noted that no clinical symptoms of toxicity or adverse effects were observed during the study period, and that no adverse effects attributable to DHNA with respect to body weight gain; feed intake; blood urea nitrogen; or serum concentrations of creatinine, calcium, or cytokine levels were observed.

Six additional preclinical studies investigating the *in vivo* effects of DHNA on various health endpoints in male and female mice were identified (Okada *et al.*, 2004, 2006a,b, 2007; Nagata *et al.*, 2010). The authors of these studies reported beneficial effects on the endpoints investigated (*i.e.,* gastric *H. pylori* levels, survival, severity of colitis, and cecal bacterial levels) following exposure of mice to 0.1 to 6 mg DHNA/kg body weight/day for 1 to 2 weeks. Although safety endpoints were not assessed, these studies suggest that oral administration of DHNA would not cause adverse effects even in animal models of intestinal inflammation.

Furthermore, DHNA is a known precursor of vitamin K_2 compounds, which are produced endogenously by the intestinal microbiota (Bentley and Meganathan, 1983). Thus, the presence of DHNA in *P. freudenreichii* ET-3 culture (powder) does not pose a safety concern.

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[7] DHNA dose calculated using the specifications for *P. freudenreichii* ET-3 culture solution provided by Meiji (*i.e.,* 8 to 12 µg DHNA/g *P. freudenreichii* ET-3 culture solution).

IV.D.2 *In vitro* Genotoxicity Tests

Two studies were identified in which the *in vitro* genotoxic/mutagenic potential of *P. freudenreichii* ET-3 culture was examined (Uchida *et al.*, 2011a). The results of these studies (detailed in Table IV.D.2-1) indicate that *P. freudenreichii* ET-3 solid culture is not mutagenic or clastogenic at exposures up to 5 mg/plate or 10 mg/mL (respectively), and support the safety of the ingredient under the intended conditions of use.

Table IV.D.2-1 Summary of Genotoxicity Studies for *P. freudenreichii* ET-3 Culture

Reference	Assay	Model	Test article	Exposure	Findings
Uchida *et al.*, 2011a	• Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* strains TA 98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 *uvr*A	*P. freudenreichii* ET-3 solid culture dissolved in water	2.29, 6.86, 20.58, 61.73, 158.19, 555.56, 1,666.67, and 5,000 µg/plate; with or without metabolic activation	• Compound was not cytotoxic at any concentration, with or without metabolic activation • No concentration-dependent in mean number of revertant colonies (*vs.* negative control) in any strain, with or without metabolic activation[a]
Uchida *et al.*, 2011a	• Mammalian chromosomal aberration test	Chinese hamster lung fibroblast cells	*P. freudenreichii* ET-3 culture (solution)	• Continuously at 3.13, 6.25, 12.5, 25, 50, or 100 mg/mL (without metabolic activation)[b] • Pulse treatment at 3.13, 6.25, 12.5, 25, or 50 mg/mL (with or without metabolic activation)[c]	• Concentrations ≥50 mg/mL cytotoxic in cells exposed continuously • No positive responses (plates with ≥10% of cells showing chromosomal aberrations or polyploidy) or dose-dependent increases in percentage of cells with chromosomal aberrations or polyploidy (with or without metabolic activation)

[a] Mean values were slightly more than two-fold higher in *S. typhimurium* TA98 treated with 555.56 or 5,000 µg *P. freudenreichii* ET-3 solid culture per plate in the presence of S9 *versus* the negative control; however, values were only marginally higher than the control and no clear dose response relationship was observed.
[b] Cells exposed for 24 to 48 hours. Doses correspond to 0.3 to 10 mg *P. freudenreichii* ET-3 solid culture/mL
[c] Following 6 hours of pulse exposure, cells were cultured for a further 18 hours. Doses correspond to 0.3 to 5 mg *P. freudenreichii* ET-3 solid culture/mL.

IV.E Studies in Humans

Three human studies (2 published and 1 unpublished) were conducted specifically to assess the safety of the consumption of *P. freudenreichii* ET-3 culture (in tablet and solution form, respectively).

In a randomized, double-blind, crossover study, 10 healthy men and 4 healthy women (24 to 41 years of age) consumed 45 *P. freudenreichii* ET-3 culture tablets/day or placebo (unfermented product) for two 1-week intervention periods separated by a 4-week washout period (Uchida *et al.*, 2011b). Hematological, clinical chemistry, and urinalysis parameters were measured at the beginning and end of each intervention period and gastrointestinal symptoms were assessed by questionnaire. There were no significant differences with respect to any measured parameter between the *P. freudenreichii* ET-3 culture and placebo intervention periods. Symptoms reported by subjects on the gastrointestinal symptoms questionnaire included diarrhea, nausea, stomach rumbling, heartburn, loss of appetite, and abdominal pain. However, all symptoms also were reported during the washout period, and the incidences of all symptoms were similar between the active and placebo supplementation periods; thus, they were not attributable to supplementation with *P. freudenreichii* ET-3 culture tablets.

In the second published human safety study, which had an open label, uncontrolled design, 11 healthy men (30 to 56 years of age) consumed 4 *P. freudenreichii* ET-3 culture tablets/day for 13 weeks (Uchida *et al.*, 2011b). Similar to the first study, hematological, clinical chemistry, and urinalysis parameters were measured at the beginning and end of the intervention period and gastrointestinal symptoms were assessed by questionnaire. Due to partial coagulation of blood samples, hematological parameters could be assessed for only 9 subjects. Total protein, white blood cell count, hemoglobin, and MCHC decreased significantly from baseline, while mean corpuscular volume and urine pH increased from baseline. These parameters remained within normal ranges, and as they were not consistent with any clinically meaningful effect, they were deemed not to be compound-related. Gastrointestinal symptoms reported by the subjects included diarrhea, nausea, stomach rumbling, heartburn, loss of appetite, and abdominal pain; however, these symptoms were reported to occur occasionally and were attributed by study subjects to a common cold, hangover, over-eating, alcohol consumption, or antibiotic use. Thus, they were not attributed to supplementation with *P. freudenreichii* ET-3 culture tablets.

In an unpublished human study provided by Meiji, 22 subjects were given a single 300 mL dose of milk containing 4.8 g *P. freudenreichii* ET-3 culture (solution) (equivalent to 0.48 g *P. freudenreichii* ET-3 solid culture) or placebo (Uchida *et al.*, 2001 [unpublished]). Blood and urine samples were collected for analysis immediately and 1, 6, and 24 hours following the consumption of the *P. freudenreichii* ET-3 culture milk. No adverse effects with respect to hematology, serum biochemistry, urinalysis, or gastrointestinal symptoms were reported.

Several published human studies investigating the effects of *P. freudenreichii* ET-3 culture (in powder, tablet, or solution form) on various biomarkers were identified. The majority of the studies were conducted to examine the effects of *P. freudenreichii* ET-3 culture with respect to bowel habit, fecal characteristics, intestinal microbiota, gastrointestinal inflammation, and gastrointestinal symptoms in healthy adults and those with irritable bowel syndrome or ulcerative colitis. No compound-related adverse effects or worsening of gastrointestinal symptoms were reported in these studies following the consumption of doses of *P. freudenreichii* ET-3 culture equivalent to 0.11 to 2 g *P. freudenreichii* ET-3 solid culture/day (providing up to 74.6 µg DHNA/day) for 1 to 4 weeks. One of these studies included an assessment of safety endpoints, and no changes were reported in routine biochemical or urinalysis parameters following the consumption of 9 *P. freudenreichii* ET-3 culture tablets/day (equivalent to 0.6 g *P. freudenreichii* ET-3 solid culture/day) for 4 weeks by adults with ulcerative colitis (Suzuki *et al.*, 2006). These studies provide supportive evidence of the safety of *P. freudenreichii* ET-3 culture (powder) under the intended conditions of use.

The results of the human studies summarized above provide evidence that *P. freudenreichii* ET-3 culture is safe for use by humans over a wide intake range (*i.e.,* equivalent to 0.11 to 3 g *P. freudenreichii* ET-3 solid culture, or up to 283.5 µg DHNA per day); thus, the safety of *P. freudenreichii* ET-3 culture for humans, under the intended conditions of use [*i.e.,* the 90th percentile consumption of up to 2.8 g solid culture] is supported. The human studies described above are summarized in tabular format in Appendix D.

IV.F Additional Considerations

IV.F.1 Production of Vitamin K_2 by *P. freudenreichii* ET-3

As mentioned previously, DHNA is a precursor to vitamin K_2 compounds, specifically, menaquinone-9 (MK-9). Accordingly, four non-consecutive lots of *P. freudenreichii* ET-3 culture (powder) have been analyzed for vitamin K content. Phylloquillone (vitamin K_1), MK-7, and MK-9 were not detected (limit of detection = 1 µg/100 g), and MK-4 was detected at levels of only 2 to 3 µg/100 g (data not shown).

Additionally, tetrahydromenaquinone-9 [MK-9(4H)] has previously been detected in various cheeses; therefore, levels of MK-9(4H) in *P. freudenreichii* ET-3 culture (powder) were calculated. Levels of MK-9(4H) ranged from 1.03 to 1.74 µg/g (*i.e.*, less than 2 µg/g). These levels are far lower than the levels detected in various cheeses (20 to 650 µg/g) as reported by Hojo *et al.* (2007).

Based on the intended uses of *P. freudenreichii* ET-3 culture, the estimated intakes of MK-9(4H) in the total population are as follows:

Mean: 2.4 g *P. freudenreichii* ET-3 culture (powder)/day[8] x 2 µg/g = 4.8 µg/day

90th percentile: 4.2 g *P. freudenreichii* ET-3 culture (powder)/day x 2 µg/g = 8.4 µg/day

These intakes provide a small contribution to the overall dietary intakes of vitamin K in all individuals as reported by the IOM (2001).

Mean: 93.9 µg/day

90th percentile: 155 µg/day

Given the low reported levels of vitamin K in P. *freudenreichii* ET-3 culture (powder) compared to levels in cheeses, and the low estimated intakes in relation to overall dietary intake of vitamin K, no safety concern is anticipated with respect to vitamin K in the ingredient.

IV.G Summary and Basis for GRAS Conclusion

The GRAS determination for the use of *P. freudenreichii* ET-3 culture (powder) as a food ingredient is based on scientific procedures. *P. freudenreichii* ET-3 culture (powder) is intended for use at levels providing up to 15% *P. freudenreichii* ET-3 solid culture in a variety of food categories. Under the intended conditions of use, the total population all-user mean intake of *P. freudenreichii* ET-3 solid culture was estimated to be 1.6 g/person/day, or 27 mg/kg body weight/day. The heavy consumer (90th percentile) all-user intake of *P. freudenreichii* ET-3 solid culture from all proposed food-uses was estimated to be 2.8 g/person/day, or 50 mg/kg body weight/day.

P. freudenreichii ET-3 culture (powder) is manufactured by incubating *P. freudenreichii* ET-3 in a lyophilized whey culture medium, during which DHNA is secreted by *P. freudenreichii* ET-3. Potato and modified starches are added as fillers to the resulting culture solution, which is then freeze-dried and sieved to produce *P. freudenreichii* ET-3 culture (powder), comprising two thirds *P. freudenreichii* ET-3 culture and one third starch. *P. freudenreichii* ET-3 culture (powder) is manufactured in accordance with cGMP, and all raw materials, processing aids, and the final product meet appropriate food-grade specifications. Lot samples of *P. freudenreichii* ET-3 culture (powder) are routinely analyzed to verify compliance with specifications. In addition, stability tests conducted on *P. freudenreichii* ET-3 culture demonstrated that the powdered product is stable under typical storage conditions.

As the intestinal mucosal barrier is impermeable to bacteria in healthy individuals, *P. freudenreichii* ET-3 cells are not expected to be absorbed from the gastrointestinal tract.

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[8] Estimated intake of *P. freudenreichii* ET-3 culture (powder) = Estimated intake of *P. freudenreichii* ET-3 solid culture x 1.5 (the powder product contains 2/3 *P. freudenreichii* ET-3 solid culture and 1/3 potato and modified starches)

Instead, the cells (most of which are killed during the final heating step of the manufacturing process) are expected to be excreted in the feces.

DHNA appears to be largely resistant to digestion under the acidic conditions of the stomach. The low molecular weight of DHNA favors its absorption *via* the gastrointestinal tract. The results of studies conducted on a compound similar in structure to DHNA suggest that DHNA will be absorbed following oral exposure and may be eliminated rapidly.

The safety of *P. freudenreichii* ET-3 culture (powder) is supported by the results of several animal studies that demonstrated that the administration of *P. freudenreichii* ET-3 solid culture to male and female rats at doses of 250 to 6,000 mg/kg body weight/day for up to 28 days was not associated with any adverse effects attributable to the ingredient (Nishimura *et al.*, 1999 [unpublished]; Uchida and Mogami, 2005; Uchida *et al.*, 2011a). In the study of the highest dose and longest duration, reported by Uchida *et al.* (2011a), male and female Sprague-Dawley rats were administered 6,000 mg *P. freudenreichii* ET-3 solid culture/kg body weight/day by gavage for 28 days. No deaths, signs of toxicity, or biologically significant hematological, biochemical, urinary, histopathological, or gross pathological findings associated with the consumption of *P. freudenreichii* ET-3 solid culture were reported. In a study in which the safety of DHNA was assessed, no compound-related adverse effects were observed with respect to systemic toxicity, body weight gain, feed intake, or blood biochemistry following the administration of 1 mg DHNA/kg body weight/day to immunosuppressed male ICR mice for 5 weeks (Matsubara *et al.*, 2010). In several other studies, in which doses up to 6 mg DHNA/kg body weight/day were administered to male and female mice by gavage for 1 to 2 weeks, no compound-related adverse effects were reported.

The safety of *P. freudenreichii* ET-3 culture is corroborated by the results of mutagenicity and genotoxicity studies. No mutagenic or clastogenic effects were observed in the bacterial reverse mutation assay, or in Chinese hamster lung cells, in the presence or absence of metabolic activation (Uchida *et al.*, 2011a).

Three human studies (2 published and 1 unpublished) were conducted to assess the safety of the consumption of *P. freudenreichii* ET-3 culture (in tablet and solution form, respectively). The published studies assessed the safety of the consumption of *P. freudenreichii* ET-3 culture tablets at levels of 3 g *P. freudenreichii* ET-3 solid culture for 1 week, or 0.267 g for 13 weeks. In these studies, no biologically significant differences in hematological or biochemical parameters between groups given *P. freudenreichii* ET-3 culture tablets and those given placebo were observed, and no gastrointestinal symptoms attributable to supplementation with *P. freudenreichii* ET-3 culture tablets were reported. Findings of the unpublished study also support the safety of *P. freudenreichii* ET-3 solid culture at a dose of 480 mg.

Results of several human studies conducted in healthy adults and those with IBS provide supportive evidence of the safety of *P. freudenreichii* ET-3 culture (powder) under the intended

conditions of use. One of these studies included an assessment of safety endpoints, and no changes were reported in routine biochemical or urinalysis parameters following the consumption of 9 *P. freudenreichii* ET-3 culture tablets/day (equivalent to 0.6 g *P. freudenreichii* ET-3 solid culture/day) for 4 weeks by adults with ulcerative colitis (Suzuki *et al.*, 2006). In the remaining studies, no adverse effects and no worsening of bowel habit, associated symptoms, or fecal characteristics were reported following the consumption of *P. freudenreichii* ET-3 culture at doses equivalent to 0.11 to 2 g *P. freudenreichii* ET-3 solid culture/day for 1 to 4 weeks.

Safety is further supported by the documented history of safe use of *P. freudenreichii* in food and the commercial availability of *P. freudenreichii* ET-3 culture products in Japan. *P. freudenreichii* has been granted QPS status by EFSA (EFSA, 2007). In addition, the International Dairy Federation, in collaboration with the European Food and Feed Cultures Association, has included *P. freudenreichii* on its inventory of microorganisms with a documented history of use in human food without adverse effects (Mogensen *et al.*, 2002). Meile *et al.* (2008) concluded in their safety assessment of dairy microorganisms that *Propionibacteria*, including *P. freudenreichii*, and *Bifidobacterium* species in dairy foods generally do not present any significant health hazards.

Together, the data provided above support the conclusion that the consumption of *P. freudenreichii* ET-3 culture (powder) under the intended conditions of use would not be expected to produce adverse effects in consumers.

Finally, the Expert Panel convened on behalf of Meiji, independently and collectively, critically evaluated the data and information summarized above and concluded that the intended uses of *P. freudenreichii* ET-3 culture (powder), produced in accordance with cGMP and meeting appropriate food-grade specifications, are safe and suitable. Furthermore, the Expert Panel unanimously concluded that the intended uses of *P. freudenreichii* ET-3 culture (powder) are GRAS based on scientific procedures. It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, Meiji has concluded that *P. freudenreichii* ET-3 culture (powder) is GRAS under the intended conditions of use on the basis of scientific procedures; therefore, the ingredient is excluded from the definition of a food additive and thus may be marketed and sold for the uses designated above in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
170—Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
172—Food additives permitted for direct addition to food for human consumption	172.854	Polyglycerol esters of fatty acids.
	172.859	Sucrose fatty acid esters.
	172.892	Food starch-modified.
182—Substances generally recognized as safe	182.3731	Sodium ascorbate
184—Direct food substances affirmed as generally recognized as safe	184.1143	Ammonium sulfate
	184.1619	Potassium carbonate
	184.1979	Whey
	184.1983	Bakers yeast extract

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APPENDIX A

Appendix A

Expert Panel Consensus Statement Concerning the GRAS Status of *Propionibacterium freudenreichii* ET-3 culture (powder) for Use in Foods

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of *Propionibacterium freudenreichii* ET-3 Culture (Powder) for Use in Foods

June 16, 2011

At the request of Meiji Co. Ltd. (Meiji), an Expert Panel (the "Expert Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened (June 16, 2011), to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether, under the conditions of intended for use as a food ingredient, Meiji's *Propionibacterium freudenreichii* ET-3 culture (powder) would be Generally Recognized as Safe (GRAS), based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Professor Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Professor John A. Thomas, Ph.D. (Indiana University School of Medicine), and Professor Eric A. Johnson, Sc.D. (University of Wisconsin-Madison).

The Expert Panel, independently and collectively, critically examined a comprehensive package of scientific information and data pertinent to *P. freudenreichii* ET-3 culture (powder) compiled from the literature and other published sources through May, 2011. This information was presented in a dossier [Documentation Supporting the Evaluation of *Propionibacterium freudenreichii* ET-3 culture (powder) as Generally Recognized as Safe (GRAS) for Use in Foods] that was submitted by Meiji to the Expert Panel. In addition, the Expert Panel evaluated other information deemed appropriate or necessary, including data and information provided by Meiji. The data evaluated by the Expert Panel included information pertaining to the method of manufacture, product specifications and analytical data, the conditions of intended use of *P. freudenreichii* ET-3 culture (powder), consumption estimates for all intended uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of *P. freudenreichii* ET-3 culture.

Following independent and collaborative critical evaluation of such data and information, the Expert Panel unanimously concluded that under the conditions of intended use as a food ingredient described herein, *P. freudenreichii* ET-3 culture (powder), meeting appropriate food-grade specifications and manufactured in accordance with current good manufacturing practice (cGMP), is GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion, excluding confidential data and information, is provided below.

SUMMARY AND BASIS FOR GRAS

Meiji intends to market *P. freudenreichii* ET-3 culture (powder), which is produced *via* the fermentation of whey with *P. freudenreichii* ET-3, as an ingredient in traditional food products in the United States (U.S.). In Japan, *P. freudenreichii* ET-3 culture tablets and milk beverages have been commercially available since 2001, and have been granted Foods for Specified Health Uses (FOSHU) status. Additionally, *P. freudenreichii* ET-3 culture (powder) has been commercially available in Japan since 2003 (MHLW, 2007[1]).

P. freudenreichii is permitted for use in the U.S. as a starter culture in the production of Swiss, Emmentaler, and Gruyere cheeses (Meile *et al.*, 2008). In addition, the U.S. Food and Drug Administration (FDA) has issued letters of no objection in response to 2 GRAS Notices pertaining to anti-microbial agents produced *via* the fermentation of corn, cane, or beet sugar, skim milk, or dextrose by *P. freudenreichii shermanii* (GRN 128, 240) (U.S. FDA, 2003, 2008).

P. freudenreichii ET-3 culture (powder) is manufactured by incubating *P. freudenreichii* ET-3 in a lyophilized whey culture medium, during which 1,4-dihydroxy-2-naphthoic acid (DHNA)[2] is secreted by *P. freudenreichii* ET-3. Potato and modified starches are added as fillers to the resulting culture solution, which is then freeze-dried and sieved to produce *P. freudenreichii* ET-3 culture (powder), comprising two thirds *P. freudenreichii* ET-3 culture and one third starch. The analysis of 4 non-consecutive lots of *P. freudenreichii* ET-3 culture (powder) demonstrated that the manufacturing process produces a consistent product that meets physical, chemical, and microbiological specifications[3]. In addition, stability tests conducted on *P. freudenreichii* ET-3 culture demonstrated that the powdered product is stable and DHNA content is consistent for up to 14 months at room temperature, and up to 7 months at temperatures of 10, 30, and 55°C. Results from stability testing also indicate that the DHNA content of *P. freudenreichii* ET-3 culture tablets is stable for 12 months at 30 or 40°C or for 15 months at 25°C.

Consumption data and information pertaining to the individual proposed food-uses of *P. freudenreichii* ET-3 solid culture were used to estimate the all-person and all-user intakes of *P. freudenreichii* ET-3 solid culture for specific demographic groups and for the total U.S. population. This type of intake methodology is generally considered to be "worst case" (*i.e.*, more likely to err on the side of overestimation, rather than underestimation, of actual

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[1] MHLW (2007). *Food for Specified Health Uses (FOSHU).* Tokyo, Japan: Ministry of Health, Labor and Welfare, Japan (MHLW). Available at: http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html.

[2] DHNA is considered by Meiji to be the component responsible for the bifidogenic activity of *P. freudenreichii* ET-3 culture.

[3] Product specifications for *P. freudenreichii* ET-3 culture (powder) are provided in Appendix A.

intakes) as a result of several conservative assumptions made in the consumption estimates.

Under the conditions of intended use in foods at levels of 200 to 600 mg *P. freudenreichii* ET-3 solid culture/serving [providing 4 to 12 µg DHNA/serving, and equivalent to 300 to 900 mg *P. freudenreichii* ET-3 culture (powder)/serving], the total population all-user mean intake of *P. freudenreichii* ET-3 solid culture was estimated to be 1.6 g/person/day [providing 32 to 144 µg DHNA/person/day and equivalent to 2.4 g *P. freudenreichii* ET-3 culture (powder)/person/day] or 27 mg/kg body weight/day [0.53 to 2.4 µg DHNA/kg body weight/day or 40 mg *P. freudenreichii* ET-3 culture (powder)/kg body weight/day]. The heavy consumer (90th percentile) all-user intake of *P. freudenreichii* ET-3 solid culture from all proposed food-uses was estimated to be 2.8 g/person/day [providing 56 to 252 µg DHNA/ person/day and equivalent to 4.2 g *P. freudenreichii* ET-3 culture (powder)/person/day] or 50 mg/kg body weight/day [0.93 to 4.2 µg DHNA/kg body weight/day or 75 mg *P. freudenreichii* ET-3 culture (powder)/kg body weight/day]. On an individual population basis, the greatest mean and 90th percentile all-user intakes of *P. freudenreichii* ET-3 solid culture on an absolute basis were determined to occur in male adults, at 2.0 and 3.3 g/ person/day [providing 40 to 180 and 66 to 297 µg DHNA/person/day, or 3 and 4.9 g *P. freudenreichii* ET-3 culture (powder)/person/day], or 23 and 39 mg/kg body weight/day [0.67 to 3.0 and 1.1 to 4.9 µg DHNA/kg body weight/day, or 34.5 and 58.5 mg *P. freudenreichii* ET-3 culture (powder)/kg body weight/day], respectively.

As the intestinal mucosal barrier is impermeable to bacteria in healthy individuals, *P. freudenreichii* ET-3 cells are not expected to be absorbed from the gastrointestinal tract. Instead, the cells (most of which are killed during the final heating step of the manufacturing process, with <300 viable cells/g remaining in the final product) are expected to be excreted in the feces.

DHNA, which is produced during the fermentation process, appears to be largely resistant to digestion under the acidic conditions of the stomach. The low molecular weight of DHNA favors absorption *via* the gastrointestinal tract. The observation of elevated plasma concentrations of 1-hydroxy-2-naphthoic acid (a monohydroxylated naphthoic acid similar in structure to DHNA) following oral exposure in humans provides support for the absorption of DHNA following oral exposure (Chilton *et al.*, 1995). The observed excretion of the majority of an oral dose of 1-hydroxy-2-naphthoic acid within 24 hours of exposure (based on the observation of minimum plasma concentrations of 1-hydroxy-2-naphthoic acid in human plasma 24 hours following oral exposure) suggests that DHNA also may be eliminated shortly following consumption (Chilton *et al.*, 1995).

The results of several animal studies demonstrated that the administration of *P. freudenreichii* ET-3 solid culture to male and female rats at doses of 250 to 6,000 mg/kg body weight/day (*i.e.,* up to 576 µg DHNA/kg body weight/day) for up to 28 days was not

associated with any adverse effects attributable to the ingredient [Uchida *et al.*, 2011; Uchida and Mogami, 2005; Nishimura *et al.,* 1999 (unpublished)]. In the study of the highest dose and longest duration, reported by Uchida *et al.* (2011), male and female Sprague-Dawley rats were administered 6,000 mg *P. freudenreichii* ET-3 solid culture/kg body weight/day by gavage for 28 days. No deaths, signs of toxicity, or biologically significant hematological, biochemical, urinary, histopathological, or gross pathological findings associated with the consumption of *P. freudenreichii* ET-3 solid culture were reported. In a study in which the safety of DHNA was assessed, no compound-related adverse effects were observed with respect to systemic toxicity, body weight gain, feed intake, or blood biochemistry following the administration of 1 mg DHNA/kg body weight/day to immunosuppressed male ICR mice for 5 weeks (Matsubara *et al.*, 2010). In several other studies, in which doses up to 6 mg DHNA/kg body weight/day were administered to male and female mice (C57BJ/6J, C57/BL6, C57BL/6NCrj, or IL-10 knockout strains) by gavage for 1 to 2 weeks, no compound-related adverse effects were reported

The safety of *P. freudenreichii* ET-3 culture is corroborated by the results of mutagenicity and genotoxicity studies. No mutagenic or clastogenic effects were observed following the exposure of several *Salmonella typhimurium* strains or *Escherichia coli* WP2 uvrA to *P. freudenreichii* ET-3 solid culture at concentrations up to 5,000 µg/plate (*i.e.,* up to 0.48 µg DHNA/plate) in the presence or absence of metabolic activation, or Chinese hamster lung cells to *P. freudenreichii* ET-3 culture (solution) at concentrations up to 100 mg/mL (*i.e.,* up to 1.02 µg DHNA/mL and 10 mg *P. freudenreichii* ET-3 solid culture).

Uchida *et al.* (2010) evaluated the safety of *P. freudenreichii* ET-3 culture tablets in two separate human studies. In a randomized, double blind, crossover study, 10 healthy men and 4 healthy women consumed 45 *P. freudenreichii* ET-3 culture tablets per day (equivalent to 3 g *P. freudenreichii* ET-3 solid culture) for 1 week, and no compound-related adverse effects on hematology, blood chemistry, or urinalysis were reported. In the second study (which had an open label, uncontrolled design), 11 healthy men consumed 4 *P. freudenreichii* ET-3 culture tablets/day (equivalent to 0.27 g *P. freudenreichii* ET-3 solid culture) for 13 weeks, and no compound-related adverse effects were reported on the same parameters evaluated in the earlier study. In an unpublished study in 22 healthy humans, Uchida, *et al.* (2001) reported no adverse effects following the consumption of a single dose of *P. freudenreichii* ET-3 culture (solution) equivalent to 0.48 g *P. freudenreichii* ET-3 solid culture.

Several human studies were conducted to assess the effects of *P. freudenreichii* ET-3 culture on bowel habit, associated discomfort, and fecal characteristics in healthy adults and those with irritable bowel syndrome or ulcerative colitis. No compound-related adverse effects or worsening of gastrointestinal symptoms were reported in these studies following the consumption of doses of *P. freudenreichii* ET-3 culture equivalent to 0.11 to 2 g *P.*

freudenreichii ET-3 solid culture/day for 1 to 4 weeks. One of these studies included an assessment of safety endpoints, and no changes were reported in routine biochemical or urinalysis parameters following the consumption of 9 *P. freudenreichii* ET-3 culture tablets/day (equivalent to 0.6 g *P. freudenreichii* ET-3 solid culture/day) for 4 weeks by adults with ulcerative colitis (Suzuki *et al.*, 2006). These studies provide supportive evidence of the safety of *P. freudenreichii* ET-3 culture (powder) under the intended conditions of use.

Safety is further supported by the documented history of safe use of *P. freudenreichii* in food, as propionic acid bacteria, including *P. freudenreichii* ET-3, are commonly used in the production of Swiss-type cheeses. As mentioned, *P. freudenreichii* appears on the inventory of microorganisms with a documented history of use in human food without adverse effects (Mogensen *et al.*, 2002) and has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (EFSA, 2007). In addition, the International Dairy Federation, in collaboration with the European Food and Feed Cultures Association, has included *P. freudenreichii* on its inventory of microorganisms with a documented history of use in human food without adverse effects (Mogensen *et al.*, 2002). Meile *et al.* (2008) concluded in their safety assessment of dairy microorganisms that propionibacteria, including *P. freudenreichii*, and bifidobacterium species in dairy foods generally do not present any significant health hazards.

CONCLUSION

We, the Expert Panel, have independently and collectively critically evaluated the data and information summarized above and conclude that the intended uses in food of *Propionibacterium freudenreichii* ET-3 culture (powder), meeting appropriate food grade specifications presented in the supporting dossier [Documentation Supporting the Evaluation of *Propionibacterium freudenreichii* ET-3 culture (powder) as Generally Recognized as Safe (GRAS) for Use in Foods], and produced in accordance with cGMP, are safe and suitable, and are GRAS based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

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Virginia Commonwealth University School of Medicine

21 June 2011
Date

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22 June 2011
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11 July 2011
Date

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U.S. FDA (2008). *Agency Response Letter GRAS Notice No. GRN 000240 [Corn, cane, or beet sugar cultured with Lactobacillus paracasei subsp. paracasei, Bacillus coagulans LA-1, or Propionibacterium freudenreichii subsp. shermanii, or mixtures of these microorganisms].* Submitted by Gorinchem, The Netherlands: Purac to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm153929.htm.

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Appendix A

Table A-1 Physical, Chemical, and Microbiological Specifications for *P. freudenreichii* ET-3 culture (powder)

Specification	Specification Parameters	Method
Composition		
Taste	No abnormal taste or odor	Organoleptic
Color	Yellow to reddish-brown	Visual
Moisture	≤8.0%	Food Sanitation Inspection Guidelines in Japan (dry at 98°C for 5 hours)
Protein	10.0 ± 2.0%	Food Sanitation Inspection Guidelines in Japan (Kjeldahl method; conversion factor: 6.25)
Ash	16.0 ± 3.0%[a]	Food Sanitation Inspection Guidelines in Japan (dry at 550°C for 5 hours)
DHNA concentration	20 to 90 µg/g	HPLC
Purity		
Lead	≤1 ppm	Food Sanitation Inspection Guidelines in Japan (AAS)
Microbial Specifications		
Total viable bacteria count	≤10,000 CFU/g	Food Sanitation Inspection Guidelines Standard methods in Japan (SMA)
Coliforms	Negative	Food Sanitation Inspection Guidelines in Japan (BGLB method)
Staphylococcus aureus	Negative	Food Sanitation Inspection Guidelines in Japan (Mannitol salt agar)
Fungi and yeast	≤1,000 CFU/g	Food Sanitation Inspection Guidelines in Japan (Potato dextrose agar)
Salmonella sp.	Negative	AOAC method (17.09.02 *Salmonella* in processed foods 967.26)

AAS = atomic absorption spectrophotometry; AOAC = Association of Official Analytical Chemists (AOAC, 2000[4]); BGLB = brilliant green lactose bile; CFU = colony-forming units; HPLC = high performance liquid chromatography; SMA = standard method agar.
[a] Meiji has indicated that the remainder of the weight of *P. freudenreichii* ET-3 culture (powder) (*i.e.*, the remaining 66 to 71% after accounting for moisture, protein, and ash content) is composed of primarily of carbohydrates, with lipids contributing approximately 0.5%.

[4] AOAC (2000). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 17th edition (2002, Revision 1)*. Arlington (VA): Association of Official Analytical Chemists (AOAC).

APPENDIX B

Appendix B

Certificates of Analysis for *Propionibacterium freudenreichii* ET-3 culture (powder)

Certificate of Analysis

June 23, 2010

Attn: Ms Ohira, Testing & Analysis G

Quality and Safety Evaluation Center, The Food Engineering Institute

Meiji Dairies Corporation

Ikedatohka Industries Co., Ltd.

(Quality Control Division, Joetsu Foods Co., Ltd.)

Seal	Supervisor
(b) (4)	

Product Name: BGS Bulk Powder (whey fermentation product)

Lot No.: 100611-01-15

Ingredient Lot No.: 100203

Parameter	Test Result	Specified Value
Taste	Good	Good
Color	Good	Good
Impurities	No anomalies	No anomalies
Moisture	0.6	≤4%
Protein	10.7	10.0±1.0%
Ash	14.1	16.0±2.0%
Arsenic (as As_2O_3)	(-)	≤1ppm
Heavy Metals (as Pb)	(-)	≤10ppm
Sodium	820	1000±200mg per 100g
Total Viable Bacteria Count	20	≤1000cfu/g
Coliform Population	Negative	Negative
Fungus/ Yeast	<5cfu/g	≤100cfu/g
Bacillus Cereus	Negative	Negative
Staphylococcus Aureus	Negative	Negative
DHNA *	70 μ g/g	20-90 μ g/g
Remarks: * Measured at Meiji Dairies Corp.		

Certificate of Analysis

August 24, 2010

Attn: Ms Ohira, Testing & Analysis G

Quality and Safety Evaluation Center, The Food Engineering Institute

Meiji Dairies Corporation

Ikedatohka Industries Co., Ltd.

(Quality Control Division, Joetsu Foods Co., Ltd.)

Seal	Supervisor
(b) (4) [redacted]	

Product Name: BGS Bulk Powder (whey fermentation product)

Lot No.: 100730-01-15

Ingredient Lot No.: 100317

Parameter	Test Result	Specified Value
Taste	Good	Good
Color	Good	Good
Impurities	No anomalies	No anomalies
Moisture	0.6	≤4%
Protein	10.1	10.0±1.0%
Ash	14.9	16.0±2.0%
Arsenic (as As_2O_3)	(-)	≤1ppm
Heavy Metals (as Pb)	(-)	≤10ppm
Sodium	(-)	1000±200mg per 100g
Total Viable Bacteria Count	5	≤1000cfu/g
Coliform Population	Negative	Negative
Fungus/ Yeast	<5cfu/g	≤100cfu/g
Bacillus Cereus	Negative	Negative
Staphylococcus Aureus	Negative	Negative
DHNA *	65 μ g/g	20-90 μ g/g
Remarks: Remarks: * Measured at Meiji Dairies Corp.		

Certificate of Analysis

November 18, 2010

Attn: Ms Ohira, Testing & Analysis G

Quality and Safety Evaluation Center, The Food Engineering Institute

Meiji Dairies Corporation

Ikedatohka Industries Co., Ltd.

(Quality Control Division, Joetsu Foods Co., Ltd.)

Seal	Supervisor
(b) (4) [redacted]	

Product Name: No. 7 BGS Bulk Powder (whey fermentation product)

Lot No.: 101102-01-15

Ingredient Lot No.: 100630/100811

Parameter	Test Result	Specified Value
Taste	Good	Good
Color	Good	Good
Impurities	No anomalies	No anomalies
Moisture	1.3	≤4%
Protein	10.9	10.0±1.0%
Ash	15.3	16.0±2.0%
Arsenic (as As_2O_3)	(-)	≤1ppm
Heavy Metals (as Pb)	(-)	≤10ppm
Sodium	(-)	1000±200mg per 100g
Total Viable Bacteria Count	80	≤1000cfu/g
Coliform Population	Negative	Negative
Fungus/ Yeast	<5cfu/g	≤100cfu/g
Bacillus Cereus	Negative	Negative
Staphylococcus Aureus	Negative	Negative
DHNA *	66 μ g/g	20-90 μ g/g
Remarks: Remarks: * Measured at Meiji Dairies Corp.		

Certificate of Analysis

January 12, 2011

Attn: Ms Ohira, Testing & Analysis G

Quality and Safety Evaluation Center, The Food Engineering Institute

Meiji Dairies Corporation

Ikedatohka Industries Co., Ltd.

(Quality Control Division, Joetsu Foods Co., Ltd.)

Seal	Supervisor
(b) (4)	

Product Name: BGS Bulk Powder (whey fermentation product)

Lot No.: 100329-01-14

Ingredient Lot No.: 091202

Parameter	Test Result	Specified Value
Taste	Good	Good
Color	Good	Good
Impurities	No anomalies	No anomalies
Moisture	1.9	≤4%
Protein	9.8	10.0±1.0%
Ash	14.3	16.0±2.0%
Arsenic (as As_2O_3)	(-)	≤1ppm
Heavy Metals (as Pb)	(-)	≤10ppm
Sodium	(-)	1000±200mg per 100g
Total Viable Bacteria Count	50	≤1000cfu/g
Coliform Population	Negative	Negative
Fungus/ Yeast	<5cfu/g	≤100cfu/g
Bacillus Cereus	Negative	Negative
Staphylococcus Aureus	Negative	Negative
DHNA *	81 μ g/g	20-90 μ g/g
Remarks: * Measured at Meiji Dairies Corp.		

Certificate of Analysis

No. 11008030001-01
February 8, 2011

Requested by: Meiji Dairies Corporation

Test Sample: Bulk Powder, Lot 329

Japan Food Research Laboratories
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo

The results of analysis performed on the above-mentioned test sample submitted to JFRL on January 28, 2011 are as follows.

Analysis Results

Analyte	Results	LOQ	Note	Method
Lead	Not detected	0.05 ppm		Atomic absorption spectrophotometry
Salmonella	Negative/25g	-	1	-

1. AOAC International (18th Edition) 967.26 [Salmonella in Processed Foods].

Any additions or modifications to this COA require the approval of Japan Food Research Laboratories.

000052

Certificate of Analysis

No. 11008030002-01
February 8, 2011

Requested by: Meiji Dairies Corporation

Test Sample: Bulk Powder, Lot 611

Japan Food Research Laboratories
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo

The results of analysis performed on the above-mentioned test sample submitted to JFRL on January 28, 2011 are as follows.

Analysis Results

Analyte	Results	LOQ	Note	Method
Lead	Not detected	0.05 ppm		Atomic absorption spectrophotometry
Salmonella	Negative/25g	-	1	-

1. AOAC International (18th Edition) 967.26 [Salmonella in Processed Foods].

Any additions or modifications to this COA require the approval of Japan Food Research Laboratories.

000053

Certificate of Analysis

No. 11008030003-01
February 8, 2011

Requested by: Meiji Dairies Corporation

Test Sample: Bulk Powder, Lot 730

Japan Food Research Laboratories
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo

The results of analysis performed on the above-mentioned test sample submitted to JFRL on January 28, 2011 are as follows.

Analysis Results

Analyte	Results	LOQ	Note	Method
Lead	Not detected	0.05 ppm		Atomic absorption spectrophotometry
Salmonella	Negative/25g	-	1	-

1. AOAC International (18th Edition) 967.26 [Salmonella in Processed Foods].

Any additions or modifications to this COA require the approval of Japan Food Research Laboratories.

Certificate of Analysis

No. 11008030004-01
February 8, 2011

Requested by: Meiji Dairies Corporation

Test Sample: Bulk Powder, Lot 102

Japan Food Research Laboratories
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo

The results of analysis performed on the above-mentioned test sample submitted to JFRL on January 28, 2011 are as follows.

Analysis Results

Analyte	Results	LOQ	Note	Method
Lead	Not detected	0.05 ppm		Atomic absorption spectrophotometry
Salmonella	Negative/25g	-	1	-

1. AOAC International (18th Edition) 967.26 [Salmonella in Processed Foods].

Any additions or modifications to this COA require the approval of Japan Food Research Laboratories.

No. 11008030 – Attachment: Analytical Procedure Flowchart

Lead (Pb)

– Atomic Absorption Spectrophotometry –

Test samples: BGS Bulk Powder; Lot Nos. 329, 611, 730, and 102

Place 5 g of BGS Bulk Powder in a Kjeldahl flask.

Add 20 mL of nitric acid and 5 mL of sulfuric acid.

Hydrolysis

Cooling

Add 10 mL of 20% hydrochloric acid.

Boiling

Transfer to a 200-mL separator.

Add 10 mL of a 50% solution of ammonium citrate dibasic.

Neutralize with ammonia water.

Indicator: Thymol blue

Cooling

Add water to make about 100 mL.

Add 5 mL of a 3% solution of ammonium pyrrolidine dithiocarbamate.

Allow the mixture to stand for 5 minutes.

Extracting

Add 5 mL of butyl acetate.

Shake for 5 minutes.

Separate the butyl acetate layer.

Perform the test as directed under Atomic Absorption Spectrophotometry.

Operating conditions for flame atomic absorption spectrophotometer

Model: Z-2310 [Hitachi High-Technologies Corporation]

Lamp: Lead hollow-cathode lamp

Wavelength: 283.3 nm

Flame: Air, 15.0 L/min; acetylene, 1.0 L/min

APPENDIX C

Appendix C

Method for Determination of DHNA in *Propionibacterium freudenreichii* ET-3 culture (powder)

Assay for 1,4-dihydroxy-2-naphthoic acid (DNHA) in BGS Bulk Powder

1. Analyte

1,4-dihydroxy-2-naphthoic acid (DHNA)

2. Equipment

High-performance liquid chromatograph with ultraviolet spectrophotometer detector (HPLC-UV)

3. Reagents and Test Solutions

- 1,4-dihydroxy-2-naphthoic acid (DHNA): Sigma, DHNA content ≥ 98%
- (±)-dithiothreitol (to prevent SH group oxidation)
- L(+)-sodium ascorbate (special grade)
- Methanol (special grade)
- Methanol (HPLC grade)
- Acetic acid (special grade)
- 1% (w/v) sodium ascorbate solution (prepared as needed):

  Add 800 ml of purified water to 10 g of sodium ascorbate, dissolve, and bring to a volume of 1,000 ml in a volumetric flask.
- 5% (w/v) sodium ascorbate solution (prepared as needed):

  Add 80 ml of purified water to 5 g of sodium ascorbate, dissolve, and bring to a volume of 100 ml in a volumetric flask.
- Dithiothreitol-sodium ascorbate solution (prepared as needed):

  Add 20 mg of dithiothreitol to 200 ml of the 1% (w/v) solution of sodium ascorbate and dissolve.
- Mixture of sodium ascorbate and methanol (prepared as needed):

  Add 10 ml of the 5% (w/v) solution of sodium ascorbate to 90 ml of methanol and mix.
- Mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol (prepared as needed):

  Add 50 mg of dithiothreitol to 1 L of a mixture of methanol, 1% (w/v) sodium ascorbate solution and acetic acid (50/49/1, v/v/v) and dissolve.

4. Preparation of BGS bulk powder test solution

(1) Weigh 1.00 g of the test substance. (Weigh directly into a 25-ml volumetric flask with a funnel.)

(2) Add 15 ml of the dithiothreitol-sodium ascorbate solution and sonicate the test substance for approximately 1 minute while stirring to suspend.

(3) Add 8 ml of methanol to the test substance suspension and sonicate for about another 3 minutes.

(4) After returning the volumetric flask to room temperature by cooling in water or allowing to stand, bring to a volume of 25 ml with methanol to prepare a test substance extract.

(5) Transfer 5 ml of the test substance extract into a 10-ml centrifuge tube and centrifuge for 5 minutes at 2,000 × g (room temperature).

(6) Filter 0.5 ml of the supernatant with a PVDF filter having a 0.22 μm pore size.

(7) Collect 0.2 ml of the filtrate, add 0.6 ml of the mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol, and mix to obtain the test solution.

5. Preparation of standard solution for calibration curve plotting

5-1 Preparation of stock standard solution

(1) Weigh exactly 0.020 g of DHNA reference standard in a 10-ml beaker.

(2) Add 10 ml of the mixture of sodium ascorbate and methanol, dissolve, and transfer the entire volume to a 20-ml volumetric flask.

(3) Bring to a volume of 20 ml with the mixture of sodium ascorbate and methanol to obtain the stock standard solution (1 mg/ml). Dispense this solution into polypropylene tubes and store at -80°C. The expiration date is 6 months from preparation. Do not repeatedly freeze and thaw.

5-2 Preparation of standard solutions (prepare as needed)

(1) 10 μg/ml-DHNA standard solution: Transfer 0.1 ml of the stock standard solution to a 10-ml volumetric flask and bring to the specified volume with the mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol.

(2) 1.5 μg/ml-DHNA standard solution: Transfer 3.0 ml of the 10 μg/ml-DHNA standard solution to a 20-ml volumetric flask and bring to the specified volume with the mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol.

(3) 1.0 μg/ml-DHNA standard solution: Transfer 2.0 ml of the 10 μg/ml-DHNA standard solution to a 20-ml volumetric flask and bring to the specified volume with the mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol.

(4) 0.5 μg/ml-DHNA standard solution: Transfer 1.0 ml of the 10 μg/ml-DHNA standard solution to a 20-ml volumetric flask and bring to the specified volume with the mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol.

(5) 0.25 μg/ml-DHNA standard solution: Transfer 0.5 ml of the 10 μg/ml-DHNA standard

solution to a 20-ml volumetric flask and bring to the specified volume with the mixture of methanol, sodium ascorbate, and acetic acid with dithiothreitol.

6. HPLC conditions

- Detection wavelength : UV 254 nm
- Column : CAPCELL PAK C18 MGII (i.d. 2.0×100 mm, 3 μm: Shiseido) or equivalently performing column
- Column temperature : 45°C
- Mobile phase : Prepared by adding 50 mg of dithiothreitol to 1 L of a mixture of methanol (HPLC grade), purified water, and acetic acid (50/49/1, v/v/v) and dissolving.
- Flow rate : 0.2 ml/min
- Test substance solution injection volume: 5 μl

If an autosampler is used, set the internal temperature to 4°C.

End of document

APPENDIX D

Appendix D

Summary of Human Studies

Table D-1 Summary of Human Studies					
Reference	**Study Design and Objective**	**Subjects**	**Dose**	**Duration**	**Results**
Published Human Safety Studies					
Uchida *et al.* (2010) - Study 1	• Randomized, double-blind crossover intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture tablets on standard safety tests, gastrointestinal symptoms, and bowel habit	• 14 healthy adults • Sex distribution: 10M, 4F • Age range: 24 to 41 years	• 45 *P. freudenreichii* ET-3 culture tablets/day [providing 3 g solid culture and 283.5 µg DHNA, and equivalent to 4.5 g *P. freudenreichii* ET-3 culture (powder)] • Placebo: unfermented product (0 µg DHNA)	6-week study, in following order: 1-week intervention, 4-week washout, 1-week intervention	• No significant differences between placebo and intervention group in hematology, clinical chemistry, or urinalysis parameters, or incidence of gastrointestinal symptoms.
Uchida *et al.* (2010) - Study 2	• Uncontrolled intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture tablets on standard safety tests, gastrointestinal symptoms, and bowel habit	• 11 healthy men • Age range: 30 to 56	• 4 *P. freudenreichii* ET-3 culture tablets/day [providing 0.267 g solid culture and 22.5 µg DHNA, and equivalent to 0.36 g *P. freudenreichii* ET-3 culture (powder)]	13 weeks	• Total protein, white blood cell count, hemoglobin, and mean corpuscular hemoglobin concentration decreased significantly from baseline. • Mean corpuscular volume and urinary pH increased from baseline. • All parameters remained within normal ranges, were not attributed to *P. freudenreichii* ET-3 culture (powder) consumption, and were not consistent with any clinically meaningful effect. • Urinary occult blood reaction in 1 subject [association with *P. freudenreichii* ET-3 culture (powder) unlikely].

Table D-1 Summary of Human Studies					
Reference	**Study Design and Objective**	**Subjects**	**Dose**	**Duration**	**Results**
Additional Published Human Studies					
Kaneko, 1999	• Placebo-controlled intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture (solution) on fecal flora and bowel habit in healthy subjects	• 19 healthy adults • Sex distribution: 9M and 10F • Mean age: 35.3 years (range: 24 to 59 years)	• 90 mL lemon drink/day containing 1.1 g *P. freudenreichii* ET-3 culture (solution) [providing 0.11 g solid culture and 6.6 to 13.6 µg DHNA, and equivalent to 0.16 g *P. freudenreichii* ET-3 culture (powder)] • Placebo: 90 mL lemon drink with no *P. freudenreichii* ET-3 culture	3-week study in following order: 1-week intervention, 1-week washout, 1-week control period	• No increase in flatulence noted during intervention period.
Satomi *et al.*, 1999	• Crossover intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture (powder) on fecal characteristics	• 21 healthy males • Mean age: 42.6 years (age range: 27 to 56 years)	• 150 mL water containing 3 g *P. freudenreichii* ET-3 culture (powder)/day (providing 14.1 µg DHNA and 2 g solid culture) • Placebo: 3 g skim milk powder in 150 mL water/day	6-week study in following order: 2-week control period, 2-week intervention, 2-week control period	• Adverse effects not reported. • No worsening of fecal microbiota composition or pH noted.
Yoda *et al.*, 2001	• Non-randomized, placebo-controlled, single-blind crossover intervention • Objective: To assess the effects of a *P. freudenreichii* ET-3 culture (powder) milk drink on bowel habit and fecal characteristics	• 61 healthy Japanese females (49 analyzed for bowel habit and 10 for fecal characteristics) • Mean age: 19.8 ± 1.1 years	• 100 mL milk containing *P. freudenreichii* ET-3 culture (powder)/day [providing 0.16 g solid culture and 6.6µg DHNA, and equivalent to 0.24 g *P. freudenreichii* ET-3 culture (powder)] • Placebo: milk with no BGS activity	8-week study in following order: 2-week run-in, 2-week control period, 2-week washout, 2-week intervention	• Adverse effects not reported. • No worsening of bowel habit or fecal characteristics (upon consumption of *P. freudenreichii* ET-3 culture) noted.

Table D-1 Summary of Human Studies

Reference	Study Design and Objective	Subjects	Dose	Duration	Results
Hojo *et al.*, 2002	• Non-randomized, double-blind, placebo-controlled crossover intervention • Objective: To assess whether *P. freudenreichii* ET-3 culture tablets is bifidogenic in the human intestine and to assess the effects of *P. freudenreichii* ET-3 culture tablets on bowel habit and fecal characteristics	• 63 healthy Japanese females (41 analyzed for stool quantity and frequency, and 7 for fecal characteristics) • Mean age: 21.0 ± 4.3 years (range: 18 to 23 years)	• 3 *P. freudenreichii* ET-3 culture tablets/day (providing 0.2 g solid culture and 6.6 µg DHNA, and equivalent to 0.3 g *P. freudenreichii* ET-3 culture (powder)] • 3 placebo tablets containing lyophilized whey	8-week study in following order: 2-week run-in, 2-week control period, 2-week washout, 2-week intervention	• No adverse effects (*e.g.*, abdominal pain or flatulence) reported by subjects during study period.
Seki and Nakao, 2003	• Double-blind, placebo-controlled crossover intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture tablets on bowel habit, fecal characteristics, and abdominal discomfort	• 66 male and female Japanese adults (55 analyzed) with inclination towards constipation or discomfort during bowel movements • Mean age: 69.5 ± 4.2 years • Sex distribution: NR	• 3 *P. freudenreichii* ET-3 culture tablets/day [providing 0.2 g solid culture and 6.6 to 24.8 µg DHNA), and equivalent to 0.3 g *P. freudenreichii* ET-3 culture (powder)] • Placebo (not described)	11-week study in following order: 2-week run-in, 3-week intervention, 3-week washout, 3-week intervention	• Adverse effects not reported. • No worsening of bowel habit, fecal characteristics, or associated discomfort (upon consumption of *P. freudenreichii* ET-3 culture) noted.
Seki *et al.*, 2003a	• Double-blind, placebo-controlled crossover intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture tablets on bowel habit and discomfort and fecal characteristics	• 21 elderly Japanese adults living in a private retirement home (15 analyzed) • Sex distribution: 7 M and 8 F • Mean age: 79.1 ± 7.7 years • Defecation frequency: ≤4 times/week	• 3 *P. freudenreichii* ET-3 culture tablets/day [providing 0.2 g solid culture and 6.6 to 24.8 µg DHNA, and equivalent to 0.3 g *P. freudenreichii* ET-3 culture (powder)] • Placebo (not described)	12-week study in following order: 2-week run-in, 3-week intervention, 4-week washout, 3-week intervention	• Adverse effects not reported. • No worsening of bowel habit and associated discomfort or fecal characteristics (upon consumption of *P. freudenreichii* ET-3 culture) noted.

Table D-1 Summary of Human Studies					
Reference	**Study Design and Objective**	**Subjects**	**Dose**	**Duration**	**Results**
Seki *et al.*, 2003b	• Active comparator-controlled intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture (powder) on bowel habit and fecal characteristics	• 35 elderly subjects requiring nursing care (19 "capable of oral ingestion" and 16 "incapable of oral ingestion") • Mean age: 82 years • Sex distribution: NR	• 0.3 g *P. freudenreichii* ET-3 culture (powder; matrix not described)/day (providing 0.2 g solid culture and 6.6 to 24.8 µg DHNA) • 0.6 g *P. freudenreichii* ET-3 culture (powder; matrix not described)/day (providing 0.4 g solid culture and 13.2 to 49.7 µg DHNA)	4 weeks	• Adverse effects not reported. • No worsening of bowel habit or fecal characteristics noted.
Seki *et al.*, 2004	• Uncontrolled intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture (powder) on bowel habit and fecal characteristics	• 18 Japanese adults requiring enteral tube feeding • Mean age: 78 years (range: 64 to 102 years) • Sex distribution: NR	• 0.6 g *P. freudenreichii* ET-3 culture (powder)/day (providing 0.4 g solid culture and 13.2 to 49.7 µg DHNA/day)	4 weeks	• Adverse effects not reported. • No worsening of bowel habit or fecal characteristics noted.
Suzuki *et al.*, 2006	• Uncontrolled intervention • Objective: To determine the therapeutic effect of *P. freudenreichii* ET-3 culture tablets in patients with ulcerative colitis	• 12 adults with physician-diagnosed mild to moderate ulcerative colitis, all intolerant or unresponsive to standard treatment (5-aminosalycylic acid, sulfasalazine, or prednisolone) • Sex distribution: 7M and 5F • All drug therapies and dietary habits kept constant during study period • Mean age: 37.2 years (range: 18 to 49 years)	• 9 *P. freudenreichii* ET-3 culture tablets [providing 0.6 g solid culture and 19.8 to 74.6 µg DHNA, and equivalent to 0.9 g *P. freudenreichii* ET-3 culture (powder)]	4 weeks	• Intervention was well-tolerated. • No adverse effects observed. • No changes noted in routine biochemical or urinalysis parameters.

Table D-1 Summary of Human Studies					
Reference	**Study Design and Objective**	**Subjects**	**Dose**	**Duration**	**Results**
Amano *et al.*, 2008 (Abstract only)	• Double-blind crossover intervention • Objective: To assess the effects of *P. freudenreichii* ET-3 culture tablets on bowel habit, fecal characteristics, and associated symptoms in patients with IBS	• 12 adults with constipation-predominant IBS • Sex distribution: 3M and 9F • Age range: 27 to 77 years	• 9 *P. freudenreichii* ET-3 culture tablets [providing 0.6 g solid culture and 19.8 to 74.6 µg DHNA, and equivalent to 0.9 g *P. freudenreichii* ET-3 culture (powder)] • Placebo (not described)	12-week study in following order: 4-week intervention, 4-week washout, 4-week intervention	• Adverse effects not reported. • No worsening of bowel habit, fecal characteristics, or associated symptoms noted.
Unpublished Human Safety Studies					
Hojo *et al.*, 2000	• Non-randomized, placebo-controlled crossover intervention • Objective: To assess the effects of yogurt containing *P. freudenreichii* ET-3 culture (solution) on bowel habit and fecal characteristics	• 56 healthy Japanese adults (56 analyzed for bowel habit and 7 for fecal characteristics) • Mean age: 20.9 years (range: 18 to 24) • Sex distribution: NR	• 90 g yogurt containing 1.8 g *P. freudenreichii* ET-3 culture solution [providing 6.6 to 13.7 µg DHNA and 0.18 g solid culture, and equivalent to 0.27 g *P. freudenreichii* ET-3 culture (powder)] • Placebo: yogurt with whey powder instead of *P. freudenreichii* ET-3 culture	8-week study: 2-week run-in, 2-week intervention, 2-week washout, 2-week control period	• Adverse effects not reported. • No worsening of bowel habit or fecal characteristics (upon consumption of *P. freudenreichii* ET-3 culture) noted.
Uchida *et al.*, 2001	• Placebo-controlled intervention • To assess the safety of *P. freudenreichii* ET-3 culture milk	• 22 adult subjects • Sex distribution: NR • Age range: NR	• 300 mL milk containing 4.8 g *P. freudenreichii* ET-3 culture (solution) [providing 43.9 µg DHNA and 0.48 g solid culture, and equivalent to 0.72 g *P. freudenreichii* ET-3 culture (powder)] • Placebo (not described)	Single dose	• No adverse effects with respect to hematology, serum biochemistry, urinalysis, or gastrointestinal symptoms were observed.

BGS = *Bifidobacteria* growth stimulation; DHNA = 1,4-dihydroxy-2-naphthoic acid; IBS = irritable bowel syndrome; NR = not reported.

SUBMISSION END